THE BEHAVIOR OF THE TUBERCLE BACILLUS TOWARD FAT-DYES.*

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. V.

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The difficulty of staining the tubercle bacillus, together with its subsequent resistance to decolorization by alcohol or by acid, first recognized by Ehrlich, has been attributed to material of a fatty nature, present especially in the outer envelope. Of those who have engaged in the investigation of this acid-fast material, Bulloch and Macleod, in their résumé of previous work, state that De Schweinitz and Dorset and Klebs consider it fat—the former workers having demonstrated the existence of palmitic, arachidic, and lauric acids—while Ruppel found three kinds of fatty substance, differentiable on the basis of the solvent used for extraction, and containing fatty acids, esters, and higher alcohols; Aronson obtained by analysis from the acidified alcohol-ether extract, free fatty acid and a waxy substance, probably an alcohol. Kresling, using chloroform as an extractive, decided that the "fat" was a mixture of a neutral fat, free fatty acid, esters, and higher alcohols (lecithin and cholesterol) Bulloch and Macleod[1] themselves demonstrated that the alcohol and acid-resistance were due to the presence of an acid-fast substance, chemically an alcohol, though non-acid-proof materials decomposable into fatty acids (notably oleic, isocetinic, myristinic, and lauric), together with lipochromes, to which cultures of tubercle bacilli owe their color, were also present.

J. Lorrain Smith[2] holds that the resistance of stained fats to acid decolorization is "another argument in favor of the accepted doctrine that the 'acid-fastness' of tubercle bacilli is due to the presence of fat in their capsules," and states that ordinary bacteria

* Received for publication February 14, 1913.

[1] *Jour. Hyg.*, 1904, 4, p. 1.

[2] *Jour. Path. and Bacteriol.*, 1906, 11, p. 415.

acquire "acid-proof" properties when grown on fats. Ritchie[1] believes that the "fat" of the tubercle bacillus is really a wax containing a higher alcohol, and it is this wax to which the acid-resistance is due. He cites in opposition to this view: Fischer, who considers the Ziehl-Neelsen method of staining dependent upon the "greater substance wealth of the bacterial protoplasm and therefore on the greater absorptive power"; Helbing, who refers it to the presence of chitin, a constituent of the tubercle bacillus also mentioned by Bulloch and by Ruppel; Grimme, who claims it is due to a substance, not fat, in the cytoplasm; and Jolles, whose dictum is: "The character of the staining is due not to fatty substance alone, but to a peculiar disposition of protein and fat in the cytoplasm."

Camus and Pagniez[2] attempted to demonstrate the presence of free fatty acid in the tubercle bacillus, and to discover whether that was the cause of the acid-resistance; but while they found that the behavior of the tubercle bacillus corresponded to that of fatty acids, they confess that the method employed (treatment of the organisms with copper sub-acetate, staining with hematoxylin, decolorization with potassium ferrocyanide) is not specific for fatty acids, and further they find that not all fatty acids are acid-proof, this quality increasing with an increase in the molecular weight of the acid. Deyke[3] states that acid-fast bacilli owe this quality to the presence of fatty substances which are the special vehicle of the free fatty acids, the source of the acid-resistance. The neutral fats share only indirectly in the acid-fastness, being the cause of the difficulty in staining.

Auclair and Paris[4] oppose the view that the acid-resistance of the tubercle bacillus is a function of the fatty or waxy substance, claiming that this property belongs to all the bacillary constituents, though in differing degree. After a four months' treatment with various solvents which completely removed the fats and waxes, the bacilli were still acid-fast. The bacterial protoplasm, isolated without alteration of its chemical constitution, was found to be acid-fast.

[1] *Jour. Path. and Bacteriol.*, 1904, 10, p. 334.

[2] *La Presse méd.*, 1907, 15, p. 65.

[3] *München. med. Wchnschr.*, 1910, 57, p. 633.

[4] *Arch. de méd. exper. et d'anat. path.*, 1907, 19, p. 129.

The residue, after the removal of albuminoids and fatty materials by boiling in KOH, responded to the test for cellulose and was extremely acid-resistant, even on prolonged treatment with HNO_3 and absolute alcohol. Acid-fastness, therefore, they conclude, belongs to all the chief constituents of the bacillus individually, though there are "nuances" in this resistance of the different parts, the protoplasm possessing it in least measure, though still to a marked degree. The staining reaction of the bacillus itself is therefore the sum of the staining reactions of each of its constituents. These workers further consider the acid-resistance of the tubercle bacillus dependent upon both its chemical and its physical constitution. Chemically the three great groups of which it consists—fats and waxes, proteins, and celluloses—are each acid-fast. But this is not a sufficient explanation of the phenomenon. It is necessary to consider the physical state in which these constituents occur. All are greatly condensed, hence the dye penetrates slowly, usually with the assistance of heat, phenol, or anilin. Decolorizing agents are also slowly taken up, and the resistance to decolorization is the natural corollary of the resistance to staining. Fontes[1] states that Hammerschlag proved that in the tubercle bacillus existed a protein-like substance which was acid-resistant. Fontes himself used Auclair and Paris' method of fat extraction, testing the bacterial residue for acid-resistance after the use of each solvent. He found this residue in every instance withstood decolorization in 1:3 HNO_3. Like Bulloch and Macleod,[2] he was able to isolate an acid-resistant wax, which was chemically one of the higher alcohols, resembling cholesterol or phytosterol. He confirmed the findings of Auclair and Paris that the bacilli remain acid-fast after treatment with fat solvents.

Gibier[3] discovered that bacteria grown in mixed culture with tubercle bacilli in broth acquired a certain resistance to acid; to him this fact seemed to show that the substance which retains the aniline dye in the body of the tubercle bacillus, in spite of treatment with acid, is not intra-cellular merely, but is also present in the liquid of the culture medium whence these other bacteria absorb

[1] *Centralbl. f. Bakteriol.*, 1909, Orig. Abt. I, 49, p. 317.

[2] *Jour. Hyg.*, 1904, 4, p. 1.

[3] *Compt. rend. Soc. de biol.*, 1897, 49, p. 789.

it. Aronson[1] also believed in the existence of an extra-bacterial substance, "tubercle wax," in the form of a secretion product between the bacilli.

Very recently Benians[2] has carried out certain experiments on the relative resistance to acid and retention of the Gram stain of crushed and uncrushed bacilli, and the results lead him to believe that the quality of acid-resistance is a purely physical phenomenon peculiar to the intact organism. When the physical integrity of the bacillus is destroyed, as by crushing, a procedure which would in nowise alter the chemical relationships of the bacterium, he finds the resistance to acid disappears. This is a rather unique conception of the significance of the acid-fastness of the tubercle bacillus and Benians' work will be more fully discussed below.

In view of the almost universally accepted fact of the existence of a fatty or fatlike constituent of the tubercle bacillus, it seems reasonable to suppose that the bacilli might manifest a particular affinity for the so-called "fat-dyes." Such dyes have served several investigators for the demonstration of the presence of fatty substances in bacterial protoplasm as well as in the tissues of higher plants and in animals. Unna was probably the first to employ a fat-stain (osmic acid) for the demonstration of the presence of fatty material in the tubercle bacillus. Ritchie[3] confirmed Unna's results—finding that the cultures of the bacilli were blackened and the individuals also colored upon treatment with osmic acid. Dorset[4] experimented on the tubercle bacillus with sudan III. Attempts at staining the bacilli after extraction with alcohol and ether were "not satisfactory," but in pure culture he obtained "very satisfactory" results. His method was to apply a cold saturated solution of the dye in 80 per cent alcohol for five or ten minutes and then wash for the same length of time in several changes of 70 per cent alcohol, whereupon the characteristic beaded appearance of *B. tuberculosis* could be very readily noted. He gives a plate showing rods and granular forms, while the text states that "the germs are found stained a bright red, and the beaded appear-

[1] Ott, *Die chemische Pathologie der Tuberculose*, p. 31.

[2] *Jour. Path. and Bacteriol.*, 1912, 17, p. 199.

[3] *Jour. Path. and Bacteriol.*, 1904, 10, p. 334.

[4] *New York Med. Jour.*, 1899, 69, p. 148.

ance is very distinct." The stain withstood decolorization for two minutes in 4 per cent H_2SO_4, HNO_3, HCl, and in NH_4OH of the same strength. Dorset was further able by means of this dye to demonstrate the presence of tubercle bacilli in sputum smears, in sections of a tuberculous gland from a guinea-pig, and in sections of tuberculous lung, and gives figures of sputum smear and of lung preparation in which the bacilli are clearly visible. He decided that sudan III was a selective stain for tubercle bacilli, and proceeded to demonstrate that fact by attempting to stain various other bacteria found in sputa, as well as preparations of pure cultures of anthrax, glanders, hog cholera, typhoid, diphtheria, *Staphylococcus pyogenes aureus*, and *B. prodigiosus*, in every case with negative results. Further, he was able to distinguish, by means of this selective action of the dye for the tubercle bacillus, between it and the smegma bacillus, which also responds to the carbol-fuchsin stain; and he states that sudan III is of practical value as a stain for the recognition of tubercle bacilli where a rapid method is desired for staining organisms in tissues, and differentiating between smegma and tubercle bacilli. Ritchie,[1] repeating Dorset's experiments, was able by means of sudan III and scarlet R to stain *cultures* of tubercle bacilli, but found the individual organisms "apparently colorless." He states that "sudan III and scarlet R are dissolved by, and consequently stain, all fats, even lecithin, myelin, and lipochrome, and therefore would stain the palmitin, stearin, wax, and lecithin of the bacilli." Though these dyes serve to demonstrate the presence of fatty substance in the bacilli—without, however, indicating the specific character of this fat—Ritchie distinctly states that they do not facilitate the detection of tubercle bacilli in film preparations made from sputum, tuberculous granulation tissue, or tuberculous "pus." Nor could any stained bacilli be detected in films of a culture of *B. tuberculosis* when stained with sudan III. In two of the 13 sputa stained, and once in a tuberculous spleen from a guinea-pig, he obtained, with sudan III, red stained bodies resembling the tubercle bacillus in size and occasionally beaded; but with scarlet R his results were uniformly negative. Moreover, such success as he had with sudan III was

[1] *Jour. Path. and Bacteriol.*, 1904, 10, p. 334.

obtained by lengthening the time of staining to from one and a half to 24 hours either at room temperature or at 37° C., followed by the washing in 70 per cent alcohol, and the use of glycerol as a mount. Dahms[1] confirms Dorset's conclusion that sudan III is a specific stain for tubercle bacilli, smegma bacilli "invariably" failing to take the stain while "tubercle bacilli always appeared red after its application," though he naïvely adds: "While the total amount of experimental work performed with Dorset's method is perhaps still too small to warrant the assertion that the process is always trustworthy, yet further experimentation will probably prove that such is the case." LeDoux[2] also used the Dorset method and states that after repeated attempts he could get no results, although he has followed the directions exactly. Sputa and sections were equally negative. Fuchs[3] was unsuccessful in his attempts to stain pure cultures of tubercle bacilli by the sudan III method, and he considers this negative behavior of cultures and material from the animal body the more remarkable since "the demonstration of the tubercle bacilli in sputum by means of sudan III was always easily successful." Finally, Cowie[4] was unable, with the exact technic of Dorset, to stain the tubercle bacillus, nor did any extension of the time of exposure to the dye, even to 12 hours, produce a more favorable result. Thereupon he entered into a correspondence with Dorset, only to discover that Dorset himself had found difficulty in duplicating his earlier results, and after testing different preparations of sudan III, and learning from the manufacturers that the dye was not a pure compound and its composition varied, had been compelled to conclude that the specificity of sudan III for tubercle bacilli was a peculiarity of one particular lot of dye, and quite a matter of accident. Sata[5] succeeded in staining tubercle bacilli in frozen sections in sudan III, but states that the staining was very faint. Unna,[6] who demonstrated the presence of fatty substances in the tubercle bacillus by means of osmic acid, also investigated

[1] *Jour. Am. Med. Assn.*, 1900, 34, p. 1047.

[2] *Centralbl. f. Bakteriol.*, 1900, Orig. Abt. I, 27, p. 616.

[3] *Centralbl. f. Bakteriol.*, 1903, Orig. Abt. I, 33, p. 649.

[4] *New York Med. Jour.*, 1900, 71, p. 16.

[5] *Centralbl. f. allg. Path. u. path. Anat.*, 1900, 11, p. 97.

[6] *Centralbl. f. Bakteriol.*, 1897, Ref. Abt. I, 21, p. 938.

the staining reactions of sudan III. He states that this dye stains olein and oleic acid more readily than it does palmitic and stearic acid, and, from the staining reaction of the tubercle bacillus, infers that its fatty substance must be more nearly related to oleic acid.

J. Lorrain Smith,[1] in employing Nile blue sulfate for the staining of fat, discovered that it was possible to obtain a red stain by acid hydrolysis of the fat, and by boiling the aqueous solution of the dye with a few drops of H_2SO_4 to secure differential staining of neutral fats and fatty acids. Fats hydrolyzed and stained resisted decolorization by 20 per cent HNO_3, a peculiarity which led him to infer that "the use of acid in the carbol-fuchsin method was probably not merely to wash out the stain from all structures save tubercle bacilli, but to hydrolyze the fat of the bacillus, and hence affect the acid-fast combination of color-base and fatty acid. Further, since this combination of color-base and fatty acid is soluble in alcohol, this might be the reason why bacilli such as the smegma bacillus are acid- but not alcohol-fast; the tubercle bacillus itself being only relatively alcohol-fast." Lastly, Eisenberg[2] has used a wide range of dyes variously modified, among them the common fat-dyes, for the staining of fat in bacteria. Most of the work, however, was done on anthrax, and he does not mention the staining reactions of *B. tuberculosis*.

The present work was undertaken to learn whether "fat-dyes" penetrate and stain the tubercle bacillus or its ether extract, and, having done so, withstand decolorization with acid or alcohol, in the hope that the results might throw light on the permeability of tubercle bacilli to organic compounds, and, perhaps, prove suggestive in the attempts to develop a chemotherapy of tuberculosis. The dyes used have been chiefly the sudan series (sudan III, sudan yellow, and sudan brown), scarlet R, and Nile blue sulfate; though Bismarck brown, indulin, indophenol blue, and dimethylaminoazobenzol, as well as basic fuchsin, eosin, neutral or anilin red, Janus green, methylene blue (für Bakteria and rectifiziert nach Ehrlich) and trypan blue (this last group falling quite outside the class of fat-dyes) have also been tested. The majority of the dyes have been

[1] *Jour. Path. and Bacteriol.*, 1907, 12, p. 1.

[2] *Centralbl. f. Bakteriol.*, 1908, Orig. Abt. I, 48, p. 257; *Virchow's Arch. f. path. Anat.*, 1910, 199, p. 502.

applied in the form of saturated solutions in 70 per cent alcohol, but aqueous solutions have also been employed where indicated. Smears of human tubercle bacilli from laboratory cultures, or of the dead bacilli (the residue after filtration in tuberculin preparation), which were kindly furnished us in large quantities by Dr. Paul Lewis and by Parke, Davis & Co., both as obtained and also after being washed to free them from foreign matter, constitute the mass of material stained.

All the dyes mentioned effected mass-staining of the smear after exposure for 24 hours at room or at incubator temperature. By mass-staining is meant a macroscopic staining of the smears, or of masses of tubercle bacilli on or from culture tubes. This is practically maximum exposure, smears stained for longer periods (48, 72, or 96 hours) showing no further absorption of dye. Frequently a few minutes' exposure was sufficient for mass-staining, but the fact that a mass-stain is obtained is never an infallible, and frequently no indication whatever of the absorption of the stain on the part of the individual organisms. Neither is the fat-solubility of a dye inseparably associated with its efficiency as a bacillus stain, for some effective dyes were non-fat-soluble.

The results obtained (see Table 1) are briefly as follows:

Sudan III.—With Dorset's technic there was no staining of the individual bacilli, nor any mass-staining in the majority of cases. Upon applying the stain hot for the same length of time, there was distinct mass-staining, but while the individual organisms could be distinguished, their color differed neither in intensity nor in tone from that of the unstained smears. Fifteen minutes at room temperature produces practically the same result. Two smears stained for that length of time in a hot solution, however, not only exhibited mass-staining, but were the only smears in which sudan III undoubtedly stained the individual bacilli. In one hour, mass-staining only occurred. As was to be expected, after 24 hours' treatment with the dye, mass-staining was the rule, nine smears showing orange-red, frequently granular masses. Two smears, however, failed to take up the dye, and in no instance were there stained individuals. Though many of the slides showed granules, these failed to exhibit the beaded arrangement characteristic of tubercle

bacilli. It was impossible by means of sudan III to detect stained bacilli in smears of "pus" or in sections of tubercle from tuberculous guinea-pigs. These results are in absolute harmony with those of Ritchie, Le Doux, and Cowie cited above, as well as with Fuchs's experience with material from the animal body. On the other hand, they are directly opposed to the experience of Dahms and to Dorset's earlier work. Sudan III, then, is useless as a stain for individual organisms. Dilute solutions of sudan III gave absolutely negative results. Most of the tests have been made with concentrated solutions of a Grübler preparation of sudan III, but an Eimer and Amend, and an Elberfeldt's Farbenfabrik preparation, called "cerise orange III," have also been tried, both with negative results. Washing in alcohol failed to remove the dye from stained preparations, but 3 per cent HNO_3 caused a slight decolorization.

Sudan yellow.—In staining with sudan yellow, the character of the dye itself makes it even more difficult than with sudan III to determine when actual staining of the bacilli occurs. Even when unstained, the bacilli have a yellowish tinge, and the clear transparent yellow of the dye produces only a slight deepening of the hue. Slides exposed for 15 minutes at room temperature show faint mass-staining but no stained individuals, while smears exposed to hot dye for the same period were chiefly negative or doubtful, though on one, clearly stained bacilli were visible. Smears stained for 30 minutes at room temperature gave rather doubtful mass-staining and no individual stain. In one hour, there was mass-staining, and one case of undoubted staining of bacilli. The bacilli on the other smears stained doubtfully, though still much resembling unstained individuals. Twenty-four hours' exposure effects individual- as well as mass-staining. Only one smear stained for that length of time was recorded as negative, the others being doubtful or positive. This dye is alcohol-fast, while nitric acid merely intensifies the color.

Sudan brown.—Fifteen minutes' exposure to sudan brown, whether at room temperature or after heating the dye, gave mass-staining only. Smears treated at room temperature for 30 minutes were faintly stained *en masse*, and one showed pale brown, alcohol-

fast organisms. Smears stained for an hour, however, showed no stained individuals. In 24 hours, all smears were stained microscopically, and four showed bacilli more or less deeply stained, though on two others no trace of bacilli could be detected. In two cases, where the microscopic picture strongly suggested individual staining, this was demonstrated conclusively by treating the slide with carbol-fuchsin, whereupon a marked portion of the smear showed a distribution of fuchsin-stained organisms, corresponding to that of the brown stained bacilli. Neither 50 per cent alcohol nor 3 per cent nitric acid decolorized the stain. Because of its brownish color, it is less difficult in the case of this dye to determine whether organisms are stained or not, and in this respect sudan brown is the most satisfactory of the sudan series.

Scarlet R.—Smears stained for 15 minutes in hot dye were colored *en masse*, but individual staining was decidedly doubtful, save in the case of a single smear. Thirty minutes at room temperature is also too short a time to obtain more than a mass-stain. In an hour, however, some slides showed the organisms colored a pale pink. In the greater number of smears exposed to the dye for 24 hours, the staining was negative, and doubtful in the remainder. Some exhibited dark granules, spaced as if at the ends of a bacillus. The stain was retained after treatment with alcohol and acid. While by no means satisfactory as a stain for individual organisms, or to be at all recommended for the detection of tubercle bacilli, the above results with scarlet R are rather more favorable than the findings of Ritchie (see above), which were uniformly negative; for here, in half of the smears, the bacillus-stain was doubtful or positive, though negative in the other half. In general it appeared to offer rather more possibility of use than does sudan III.

Nile blue sulfate.—Aqueous solutions of Nile blue sulfate stain *en masse* in 15 minutes, but even with an exposure of 24 hours the individual stain is shadowy and indefinite. In no instance did the aqueous stain cause a red colorization of the organisms, either after the smear was washed with acid or upon exposure to the air after staining, a result to be expected if, as J. Lorrain Smith claims, the staining is the combination of the dye-base with the fatty acid set free by hydrolysis of the fat, either upon washing with acid or

exposure to the action of the atmospheric carbon dioxid. Of the smears stained 15 minutes in alcoholic solution of the dye, one showed mass-staining only; the others were doubtful or showed pale blue individuals. After staining for an hour, blue, shadowy bacilli were visible, while in 24 hours, all smears exhibited doubtful individual-staining except one negative smear. Of the smears stained for 96 hours, one was doubtful, while the others showed definitely stained organisms. Neither acid nor alcohol effects decolorization.

Janus green.—When allowed to act for only 15 minutes or for an hour, Janus green gives only a shadowy outline of individuals. In 24 hours, the results were about evenly balanced, some smears being unstained, and others showing pale, greenish bacilli. In a single instance definitely and sharply stained bacilli of characteristic appearance were visible. A peculiarity of smears stained in Janus green is their variegated mass-staining. It was rare to find a slide that was bluish or greenish only. The majority were greenish with violet or reddish patches, but some were entirely violet or red. Alcohol does not remove the dye, but acid does, strongly, with only a short exposure.

Neutral red.—After one hour in the dye, individual staining is still doubtful. Mass-staining occurs, and the clumps show darker granules and a very few rods. In 24 hours, some smears still showed no stained organisms, though in others were very faintly pink bacilli. The dye withstands alcohol but not acid.

Indulin.—After one hour, smears show pale organisms. After 24 hours, there is undoubted individual-staining which withstands decolorization with 50 per cent alcohol and 3 per cent HNO_3. The color of the dye makes it possible readily to differentiate stained from unstained bacilli, and in this respect it is far better than the sudans.

Indophenol blue.—Twenty-four hours in the dye gives only a faint mass- and no individual-stain.

Dimethylaminoazobenzol.—In 24 hours, a faint mass-stain, far paler than with sudan yellow, was visible, with a slightly granular individual stain. Alcohol and HNO_3 failed to decolorize.

Eosin.—Alcoholic solutions of eosin in 24 hours stain both the

TABLE 1.

ACTION OF DYE ON SMEARS OF TUBERCLE BACILLI.

DYE	SOLVENT	TIME OF EXPOSURE	TEMPERATURE	TOTAL NO. OF SLIDES	MASS-STAINING		INDIVIDUAL-STAINING			DECOLORIZATION	REMARKS
					Positive	Negative	Negative	Doubtful	Positive		
Sudan III.....	70 per cent alcohol	2–3 min.	Hot	1	1	..	..	1	..	Alcohol-fast but slightly decolorized by 3 per cent HNO_3	Granules
		5 min.	Cold	1	..	1	1	..	..		
		10 min.	Cold	3	1	2	3	..	..		
			Hot	4	4	..	4	..	..		
		15 min.	Cold	6	4	2	6	..	..		
			Hot	2	2	..	..	1	1		
		30 min.	Hot	1	1	..	..	1	..		
		1 hr.	Cold	7	7	..	7	..	..		
		24 hrs.	Incubator	8	6	2	8	..	..		Faint mass-stain granules
			Cold	3	3	..	1	2	..		
		48 hrs.	Cold	2	2	..	2	..	..		
		96 hrs.	.....	4	4	..	4	..	..		
	(Herxheimer's) KOH	24 hrs.	Cold	3	2	1	3	..	..		
Sudan yellow.....	70 per cent alcohol	15 min.	Cold	3	3	..	3	..	..	Withstands 50 per cent alcohol 3 per cent HNO_3	
			Hot	3	3	..	2	..	1		
		30 min.	Cold	2	1	1	2	..	..		
		1 hr.	Cold	3	3	..	..	2	1		
		24 hrs.	Cold	6	6	..	1	2	3		
Sudan brown.....	70 per cent alcohol	15 min.	Cold	1	1	..	1	..	..	(50 per cent) Alcohol- and acid-fast (3 per cent HNO_3)	
			Hot	2	2	..	2	..	..		
		30 min.	Cold	3	3	..	1	2	..		
		1 hr.	Cold	3	3	..	2	1	..		
		24 hrs.	Incubator	3	3	..	2	1	..		
		24 hrs.	Cold	6	6	..	..	3	3		
Scarlet R.....	70 per cent alcohol	15 min.	Hot	4	4	..	1	2	1	Alcohol- and acid-fast	
		30 min.	Hot	1	1	..	..	1	..		
		1 hr.	Cold	4	4	..	1	1	2		
		24 hrs.	Incubator	4	4	..	4	..	..		
			Cold	7	7	..	4	3	..		
Nile blue sulfate...	Aq.	15 min.	Cold	3	3	..	1	2	..	Alcohol- and acid-fast	
			Hot	3	3	..	2	1	..		
	70 per cent alcohol	15 min.	Cold	3	3	..	1	1	1		
		1 hr.	Cold	3	3	..	1	1	1		
		24 hrs.	Cold	6	6	..	1	2	3		
			Incubator	2	2	..	..	2	..		
	Aq.	24 hrs.	Incubator	3	3	..	1	2	..		
	Alc.	96 hrs.	Cold	2	2	..	..	1	1		

TABLE 1—*Continued.*

Janus green.....	70 per cent alcohol	15 min.	Cold	5	5	..	1	2	2	Alcohol-fast.	
		1 hr.	Cold	2	2	..	2	..	..	3 per cent HNO_3	
		24 hrs.	Incubator	3	3	..	2	..	1	decolorizes somewhat	
	Aq.	24 hrs.	Cold	6	6	..	2	2	2		
			Incubator	1	1	..	..	..	1		
Neutral red.....	70 per cent alcohol	1 hr.	Cold	1	1	..	..	..	1	Alcohol- but not acid-fast	Granules
		24 hrs.	Cold	4	4	..	2	1	1		
			Incubator	1	1	..	..	1	..		
Indulin.....	70 per cent alcohol	1 hr.	Cold	3	3	..	1	2	..	Alcohol- and acid-fast	Better than sudan III
		24 hrs.	Cold	3	3	..	1	..	2		
	Aq.	24 hrs.	Incubator	1	1	..	1	..	..		
Indophenol blue...	70 per cent alcohol	24 hrs.	Incubator	1	1	..	1	..	..		
Dimethylamino-azobenzol.....	70 per cent alcohol	24 hrs.	Cold	1	1	..	..	1	..		Granules
Eosin.....	70 per cent alcohol	24 hrs.	Incubator	5	5	..	..	..	5	Alcohol- and acid-fast	As satisfactory a stain as carbol-fuchsin though paler
	5 per cent phenol	30 min.	Boiling	1	1	..	..	..	1		
		24 hrs.	Incubator	2	2	..	..	..	2		
Bismarck brown...	70 per cent alcohol	2 min.	.....	1	1	..	..	..	1	Alcohol- and acid-fast	Never succeeded in duplicating time; 15 min. shortest time possible to get bacillus-stain
		15 min.	Hot	3	3	..	1	1	1		
		1 hr.	Cold	5	5	..	1	3	1		
		24 hrs.	Cold	6	6	..	..	3	3		
	5 per cent phenol	10 min.	Cold	1	1	..	..	1	..		
		1 hr.	Cold	1	1	..	..	..	1		
		24 hrs.	Cold	5	5	..	2	..	3		
		96 hrs.	Cold	2	2	..	..	..	2		
Basic fuchsin.....	70 per cent alcohol	15 min.	Cold	1	1	..	..	..	1	Alcohol- and acid-fast	
Methylene blue *f. Bakt*.....	Alcoholic	24 hrs.	Cold	1	1	..	..	..	1	Alcohol- and acid-fast	Deep mass-stain. Rather uniform individual stain, few granules
	Aq.	30 min.	Cold	1	1(heavy)	..	..	..	1 (gran.)		
Methylene blue *rect*.....	Alcoholic	24 hrs.	Cold	1	1	..	..	..	1 (unif'm)		
		30 min.	Cold	1	1	..	..	..	1 (faint granular)		
	Aq.	30 min.	Cold	1	1	..	..	..	1 (granular)		
		24 hrs.	Cold	1	1	..	..	..	1 (granular)		
Trypan blue.....	Aq.	30 min.	Cold	1	1	..	..	..	1 (faint)		

smears as a whole and the bacilli themselves, and the stain is alcohol- and acid-fast. Though paler, the coloration is quite as definite as with carbol-fuchsin, and, with the possible exception of basic fuchsin, this is by far the most satisfactory of the dyes tested.

Bismarck brown.—In the early work a smear was obtained that after two minutes' exposure to this dye showed stained bacilli, but later work has failed to duplicate this result. Fifteen minutes with heat gave pale yellow bacilli. After one hour, the results were not satisfactory, though there were shadowy organisms, and a few dark rods. In 24 hours, the individual-stain was always positive, though faint. Occasionally organisms would be beaded, but for the most part there was a uniform individual-stain. The dye is acid- and alcohol-fast.

With basic fuchsin and the two preparations of methylene blue, after an exposure of 30 minutes, as well as after 24 hours, both mass- and individual-staining were obtained, a result in accord with that of DeWitt.[1] It is interesting to note, in this connection, that, while treatment with the alcoholic solution of methylene blue *rect.* resulted in uniform individual-staining, the aqueous solution gave the beaded appearance so characteristic of the tubercle bacillus.

Some attempt was made to find whether mordanting a dye increased its penetrability. Herxheimer's modification of sudan III—saturated solution of the dye in alcohol, with the addition of 2 per cent KOH—was less satisfactory than the simple alcoholic solution. The mass-stain after 24 hours' exposure was exceedingly faint, while the individuals did not differ perceptibly, save perhaps by being slightly swollen, from the unstained. Acid and alcohol had no effect. Carbol-eosin stained the individual bacilli in 30 minutes, with heat, and in 24 hours, at incubator temperature, but the stain was paler than eosin alone gave, and the color was brownish instead of pink. Carbol-Bismarck-brown, however, stained the bacilli faintly in 10 minutes. In one hour, the organisms were pale yellow-brown. In 24 hours, all smears showed stained individuals and among this number was one of the few clear bacillus-stains obtained in this work. Ninety-six hours' exposure effected no improvement over 24 hours' staining. It is quite possible that very

[1] *Jour. Infect. Dis.*, 1913, 12, p. 68.

profitable investigation might be made in the way of modifying dyes to increase their penetrability, following the suggestions of Eisenberg,[1] as, for instance, mordanting the material to be stained, directly, by the use of an acid or an alkali, or indirectly, by linking these substances with the dye in solution, by the use of various solvents for the dye (formalin, glycerol, chloroform, etc.), by the addition of anilin or phenol to the dye, or by alterations of its configuration.

In addition to the staining of smears of dead tubercle bacilli on the slide, agar culture tubes of the living organism were filled with dye, left for 24 hours, and then washed with 50 per cent alcohol and smears made. The results were as follows:

Dye	No. of Slides	
Sudan III (alc.)	2	Mass-stain, no stained individuals.
Sudan III (Herx.)	2	" " " " "
Sudan yellow	2	" " one slide shows stained individuals, the other only dry crystals.
Sudan brown	2	Mass-stain, pale individuals.
Scarlet R	2	" " no stained individuals.
Nile blue sulfate	2	" " granular stain of individuals.
Janus green	2	" " individuals stained.
Neutral red	2	" " " well stained.
Indulin	2	" " " not well stained.
Bismarck brown	2	" " " rather deeper in color than with sudan brown.
Carbol-Bismarck-brown	2	" " yellow-brown, granular staining of individuals.

In glancing over this table there is manifest a considerable variability in the behavior of the different stains with different periods of staining, and even within the limits of the same period. There is no regularity about it. It is impossible to predict that if a dye applied for, say 15 minutes, stains the individual organisms, an hour's application will stain them more deeply, or even stain them at all; or that because one smear, stained for 24 hours, showed colored individuals, every smear stained for that length of time will do the same. To some extent this irregularity appears to be dependent upon the character of the smear itself. With dyes so uncertain in their action as most of those mentioned above—dyes which under the most favorable conditions give only pale colorings, lacking entirely the sharpness of the carbol-fuchsin stain—it is especially important that the smears be thin and

[1] *Centralbl. f. Bakteriol.*, 1908, Orig. Abt. I, 48, p. 257; *Virchow's Arch. f. path. Anat.*, 1910, 199, p. 502.

uniform, and the organisms well separated. This is of twofold importance, both that the greatest opportunity be afforded for the penetration of the dye, and because, where a clump of bacilli appears colored, it is practically impossible with these dyes to say with certainty that it is the individuals which are stained rather than the mass of fatty material. This matter of the character of the smear can hardly be the entire explanation of the irregularity met with. There seem to be involved other factors, not yet identified, against which, because of their very indefiniteness, it has been impossible to guard. On the whole, these dyes seem likely to prove of very little value as stains for the tubercle bacillus, since even when there is undoubted staining of the organisms, it is not sufficiently sharp and definite to admit of the detection of the bacilli in tissue or in pus smears, especially when these are partially stained with the same dye.

A few experiments were made in staining smears of tuberculous pus from guinea-pigs, with sudan III or scarlet R, and counterstaining with methylene blue, or using Nile blue sulfate and Bismarck brown in combination. The results were absolutely negative. The primary stain produced a diffuse color which was replaced or modified by the counterstain, giving a diffuse, undifferentiated staining of the entire smear.

With the exception of Bismarck brown, and possibly of sudan brown and Nile blue sulfate, the "fat-dyes," i.e., the sudan series, scarlet R, Nile blue sulfate, dimethylaminoazobenzol, indulin, indophenol, and Bismarck brown, which is sometimes included in that category, do not stain the tubercle bacillus nearly so distinctly as do basic fuchsin, eosin, and methylene blue. Not only are these dyes insoluble or only slightly soluble in fat,[1] but further, they are perfectly efficient in aqueous solution. Apparently a physical affinity of the bacterial fats for the fat dyes is a negligible quantity in the staining of the tubercle bacillus.

In addition to attempts at staining the entire organism, the behavior of the ether extract of the bacilli toward the various dyes was also studied. The ether extract was prepared by Dr. H. J. Corper of this laboratory, as follows: Dead tubercle bacilli,

[1] DeWitt, *Jour. Infect. Dis.*, 1913, 12, p. 68.

the tuberculin residue containing glycerol, pepton, and other foreign matter, were washed in salt solution and extracted, first with a mixture of hot absolute alcohol and ether, then with hot ether. The earlier tests were made with this impure ether extract. Later, because of the discovery that it stained with almost any dye indiscriminately, this extract was ground with sand, extracted with cold absolute ether in a Söxhelet extraction apparatus, shaken out with water to remove water-soluble substances, and dried, constituting the pure ether extract. Smears of the fat were made both on the slides and on cigarette paper. It was found that great care must be taken that these smears be of uniform thickness, since frequently the mechanical difficulties of staining a thick smear gave rise to seemingly contradictory results in the action of a dye, or led to error in the comparison of one dye with another. In the earlier work, no especial attention was paid to uniformity of exposure to the dye, but it was found that all dyes stained the extract. Later, an attempt was made to establish the minimum time necessary for staining in the case of each dye, and also, by freeing the extract from proteins or other non-fatty substance whose presence might account for the ease in staining, to insure the contact of the dye with the extract of fats solely.

The results are given in Table 2. All the dyes stained the impure ether extract. In the case of the sudans and scarlet R, one second sufficed to effect staining. Neutral red and indulin required about a minute; Bismarck brown, one to five minutes; Janus green, two to ten, and Nile blue sulfate, five to fifteen minutes. Ether extract smears on the slide stained with Janus green exhibited the variegated appearance already mentioned, the colors ranging from green to violet and red. Smears stained with Nile blue sulfate, after standing for some time, occasionally showed reddish or orange patches, a result, according to Smith,[1] of the hydrolytic action of atmospheric CO_2. Following Smith's suggestion,[2] the aqueous solution of Nile blue sulfate was boiled with a few drops of H_2SO_4, to increase the oxazone base of the dye, to whose presence is due the red coloration with neutral fats, fatty

[1] *Jour. Path. and Bacteriol.*, 1906, 11, p. 415. [2] *Ibid.*, 1907, 12, p. 1.

TABLE 2.

STAINING OF ETHER EXTRACT OF TUBERCLE BACILLI.

DYE	TIME OF EXPOSURE	IMPURE ETHER EXTRACT				PURE ETHER EXTRACT				DECOLORIZED
		SLIDE		PAPER		SLIDE		PAPER		
		Stain	Minimum Time for Definite Coloration	Stain	Minimum Time for Definite Coloration	Stain	Minimum Time	Stain	Minimum Time	
Sudan III.....	15 min.	+	..	+	..	..	..	..	..	Withstands 50 per cent alcohol and 3 per cent acid
70 per cent alcohol	1 min.	+	..	+	..	..	..	..	..	
	15 sec.	+	..	+	..	..	..	..	..	
	5 sec.	+	..	+	..	..	..	..	..	
	1 sec.	+	1 sec.	+	1 sec.	+	1 sec.	+	1 sec.	
Herxheimer's KOH..	72 hrs.	Faint	..	..	..	..	..	..	..	
Sudan yellow.....	15 min.	+	..	+	..	..	..	..	..	"
	1 min.	+	..	+	..	..	..	..	..	
	1 sec.	+	1 sec.	+	1 sec.	+clear	1 sec.	+faint	1 sec.	
Sudan brown.....	15 min.	+	..	+	..	..	..	..	..	"
	1 min.	+	..	+	..	..	..	..	..	
	1 sec.	+	1 sec.	+	1 sec.	+good	1 sec.	+good	1 sec.	
Scarlet R.....	15 min.	+	..	+	..	..	..	..	..	"
	1 min.	+	..	+	..	..	..	..	..	
	1 sec.	+	1 sec.	+	1 sec.	+faint	1 sec.	+faint but distinct	1 sec.	
Nile blue sulfate....	1 sec.	± (?) Faintly blue in thin parts of smear; color increases slightly with each immersion	..	..	..	−	..	−	..	"
	2 sec.		..	..	..	..	..	..	..	
	3–15 sec.		..	Faintly greenish tinge	..	..	..	..	..	
	1 min.	+Nearly entire smear stained faintly	..	−	..	−	..	+	..	
	2 min.	+Not uniform	..	−	..	..	..	..	..	
	3 min.	" "	..	+upon soaking	..	..	..	..	..	
	4 min.	" "	..	..	..	..	..	..	..	
	5 min.	" "	..	+not uniform	..	−	..	+	..	
	15 min.	+5–15 min.	..	..	..	−	..	..	..	
	1 hr.	..	..	..	..	..	..	..	..	
	72 hrs.	..	..	..	..	+not deeply stained +deep blue	..	..	..	

TABLE 2—*Continued.*

Janus green.....	1 sec.	..	..	..	..	−	..	−	..	Withstands 50 per cent alcohol and 3 per cent acid
	3 sec.	Not uniform	..	..	..	..	..	..	..	
	5 sec.	" "	..	..	..	..	..	..	..	
	10 sec.	" "	..	..	..	..	..	..	..	
	15 sec.	" "	..	..	..	..	..	..	..	
	30 sec.	" "	..	..	..	..	..	..	..	
	1 min.	" "	..	−	..	..	..	−	..	
	2 min.	+thin smear	..	+	..	..	..	..	..	
	5 min.	−	..	−	..	..	..	..	..	
		+faint and not uniform even on soaking	..	+	..	..	..	−	..	
	7 min.	+	..	..	..	..	..	..	..	
	10 min.	+	5–15 min.	+	10–15 min.	..	..	..	..	
	15 min.	..	..	+	..	..	..	..	..	
	1 hr.	..	..	..	..	−	..	−	..	
	2 hrs.	..	..	..	..	−	..	..	..	
	5 hrs.	..	..	..	..	−	..	..	..	
	24 hrs.	..	..	..	..	−	..	..	..	
Neutral red.....	1 sec.	−	..	..	..	−	..	−	..	"
	2 sec.	Faint	..	..	..	..	..	..	..	
	3 sec.	Not uniform	..	+	..	..	..	..	..	
	4 sec.	" "	..	..	..	..	..	..	..	
	5 sec.	+	..	..	..	..	..	..	..	
	6 sec.	+	1 min.	..	1 min.	..	..	..	..	
	1 min.	..	..	..	..	..	..	−	..	
	5 min.	..	..	..	..	..	..	+	..	
	8 min.	..	..	..	..	−	..	..	..	
	1 hr.	..	..	..	..	+	..	..	..	
Indulin.....	1 sec.	−	..	−	..	..	..	−	..	"
	2 sec.	+	2 sec.	−	..	..	..	..	..	
	3 sec.	..	..	−	..	+faint	..	..	..	
	4 sec.	..	..	?	..	..	..	..	..	
	6 sec.	..	..	+faint	..	..	..	..	..	
	1 min.	..	..	..	..	+faint	..	−	..	
	5 min.	..	..	..	..	+	..	−	..	
	15 min.	..	..	..	..	+slightly deeper	..	..	..	
	30 min.	..	..	..	..	+	..	..	..	
	1 hr.	..	..	..	..	+faint but distinct	..	+	..	

TABLE 2—*Continued.*

Dye	Time of Exposure	Impure Ether Extract				Pure Ether Extract				Decolorized
		Slide		Paper		Slide		Paper		
		Stain	Minimum Time for Definite Coloration	Stain	Minimum Time for Definite Coloration	Stain	Minimum Time	Stain	Minimum Time	
Bismarck brown....	1 sec.	−	..	..	..	−	..	+	..	Withstands 50 per cent alcohol and 3 per cent acid
	2 sec.	Faint	..	..	..	−	..	..	..	
	3 sec.	..	..	..	..	−	..	..	..	
	4 sec.	+	..	+faint	..	..	..	..	..	
	2 min.	+?	..	..	..	..	..	..	..	
	4–5 min.	+	5 min.	+	2 min.	+	5 min.	..	..	
Carbolic.....	1 sec.	..	..	..	..	−	..	−	..	"
	1 min.	..	..	..	..	−	..	+	1 min.	
	5 min.	..	..	..	..	+	5 min.	..	..	
Trypan blue aq.....	30 min.	+	..	..	..	−	..	..	..	"
Eosin.....	30 min.	Faint pink	..	..	..	..	..	..	..	"
	48 hrs.	" "	..	..	..	−	..	..	..	
Fuchsin.....	5 min.	+	..	+	..	..	..	..	..	"
	30 min.	+	..	+	..	+	..	+	..	
Methylene blue *rect.*	30 min.	+	..	..	..	−	..	..	..	"
Alcoholic	24 hrs.	−	..	..	..	+	..	..	..	
Aq.....	30 min.	−	..	..	..	−	..	..	..	
	24 hrs.	..	..	..	..	+	..	..	..	
Methylene blue *f. Bakt.* Alcoholic	30 min.	−	..	..	..	..	..	..	..	"
		+	..	..	..	−	..	..	..	
Aq.....	21 hrs.	−	..	..	..	−	..	..	..	
	30 min.	+	..	..	..	−	..	..	..	
	24hrs.	..	..	..	..	−	..	..	..	

acids staining blue with the oxazine base. Smears treated with this acidified solution showed large areas stained red, while blue staining preponderated with the use of the simple aqueous and the alcoholic solution.

There was very little difference in the behavior of the pure ether extract toward the sudans and toward scarlet R. Possibly the stain in the minimum time is a little paler, but definite staining occurs in the same interval. Bismarck brown and basic fuchsin also show the same minimum for both ether extracts. In the case of the other dyes, however, a marked lengthening of the time is necessary for the absorption of the stain. Indulin requires five minutes instead of two seconds, neutral red and Nile blue sulfate, an hour, instead of one and 15 minutes, respectively; Janus green and indulin, methylene and trypan blue no longer stain. This result seems to confirm the accuracy of the inference that it was the presence of non-fatty matter in the impure ether extract that caused it to stain so readily and so universally.

In working with Nile blue sulfate and neutral red, and to a less degree in the case of Bismarck brown, it was found that smears of ether extract on cigarette paper showed definite staining only after prolonged soaking of the dye-treated smear in water. This peculiarity first came to light in comparing the effect of these dyes on smears on the slide with that of smears on paper. The former showed a coloration of the ether extract in a given time when the latter did not. Upon investigation it was found that when the smears on paper were soaked in water for from one to several hours, the dye was removed from the paper by which it had been readily absorbed, while the coloration of the smear not only became thus visible, but was also observed to be deeper than that of smears stained in the saturated dye for the same length of time, but not soaked afterward. This might be a purely physical phenomenon, the dye passing into the smear only when the absorptive power of the paper was satisfied, and water might serve this end as well as alcohol, or there might be some other explanation. Dr. DeWitt has suggested that the soaking may set free some constituent of the dye or of the extract, thereby facilitating the combination of the two, or (and this seems the more likely) that this may be a function

of dilute solutions, the soaking in water being tantamount to the dilution of the dye, which in the concentrated solution may form a precipitate over the surface of the smear and prevent its complete penetration and coloration, or, if it is a chemical process, the complete union of the dye with the fat. This theory is strengthened by the results with dilute solutions of Nile blue sulfate and neutral red. Into two portions of about 50 c.c. of water were shaken a few crystals of the dyes, so that the resulting solutions were of the same shade as the solution of one drop of the concentrated dye in 10 c.c. of water. Four smears, two on cigarette paper and two on slides, one each of the pure and the impure ether extract, were placed in each diluted dye. In 15 minutes a faint coloration was visible. The smears were left for 24 hours, when all were deeply stained, the depth of color and sharpness of outline being precisely that of smears stained a short time in concentrated dye and then soaked in water.

In connection with the behavior of neutral red, it was noted that upon first removing the smear on paper from the concentrated dye, the paper was dull red, but after soaking, the smear was deep raspberry red. A strip of the paper was stained to see how it responded to the dye, and it was found that upon a portion which had been held between the fingers there was this same difference in color from the remainder of the strip. Inasmuch as neutral red is sometimes used as an indicator for alkali and acid, and as this color was that produced by the addition of an acid to the dye, this suggested that it was an acid that caused the color change, and specifically the fatty acid of the ether extract, which, in the dilute solution, was free to combine with the dye. The behavior of fats and fatty acids toward Nile blue sulfate and neutral red was investigated, and it was found that butyric, oleic, and stearic acids readily take up these dyes from dilute aqueous solutions (one drop of concentrated dye solution to 10 c.c. of water), forming deeply colored rings beside the paler or completely decolorized solution, thus demonstrating the fact that fatty acids can remove the dye from dilute aqueous solutions. Palmitin, dissolved in chloroform, and olive oil also took up the dye, though the olive oil took up the dye, particularly Nile blue sulfate, far less readily than did the fatty acids. The result

is not entirely in accord with the findings of Smith, who was able to stain fatty acids, but not neutral fats.

In connection with the tests already described, Benians'[1] experiments with crushed tubercle bacilli have been repeated with the fat dyes. Benians employed two methods for crushing bacteria: either grinding for half an hour in an agate mortar, or rubbing a coverslip with a grinding motion over one end of a smear after fixation upon the slide. He states that the former method is preferable, though "the results obtained by the method used for crushing fixed films is necessarily very partial and imperfect in most cases." This statement, together with those that films so crushed show under the microscope "masses of amorphous material mingled with intact bacilli" and that "the actual extent to which these bacilli are really crushed is naturally a matter of some doubt" found ample confirmation in the present instance. Although Benians' thesis, that "the bacterial substance itself does not exhibit in any marked degree the property of gram-positive staining or of acid-fastness, but both these properties are almost entirely dependent on the integrity of the cell" is directly opposed to the findings of previous workers, notably to those of Auclair and Paris, of Fontes and of Bullock and Macleod mentioned above, so far as the acid resistance of the tubercle bacillus is concerned my results are in accord with those of Benians. There is a marked loss of acid-fastness upon crushing the bacilli, as judged by the reaction toward the Ziehl-Neelsen stain and there is a change in the behavior with the fat dyes. With the exception of indulin all the dyes used in the previous work stained the uncrushed smear more deeply than the crushed, but in the case of the true fat dyes, the sudans and scarlet R, there was a further noticeable difference. Whereas the uncrushed smear showed amorphous, deeply stained masses, with here and there faintly stained objects suggesting individual bacilli, in the crushed smears, there were numerous faintly colored masses resembling clumps of undistributed bacteria, and upon these as a background were found thickly scattered, deeply staining granules, apparently once that part of the bacterial contents which caused the familiar "beaded" appearance. The occurrence of such

[1] *Jour. Path. and Bacteriol.*, 1912, 17, p. 199.

granules was especially marked on slides stained with scarlet R and with sudan brown. Crushing the tubercle bacillus, then, alters both its acid resistance and its behavior toward the fat dyes.

Dr. H. J. Corper has investigated in this laboratory the effects of fat dyes upon the growth of tubercle bacilli in culture media, and permits me to incorporate in this article the results of his experiments. In these growth experiments, 0.1 c.c., 0.5 c.c., and 1.0 c.c. of a 1 per cent solution of the fat dye in peanut oil were added to a 5 per cent glycerol agar. In the cases where 1 c.c. of the oil was used, the culture did not grow, the oil being present in sufficient amount to prevent growth; but with smaller amounts of oil, growth generally occurred at the border of the oil, and staining was easily observed there. The cultures were made in small bottles, rhomboid in shape, laid on one side.

The chief results of these experiments were as follows: In culture experiments masses of tubercle bacilli are stained macroscopically when in contact with oil containing sudan III, scarlet R, sudan yellow, dimethylaminoazobenzol, indulin, and Nile blue sulfate. The last two dyes displayed a marked toxicity toward the organism. Bismarck brown when suspended in oil does not appreciably stain the cultures, but when dissolved in the agar does stain the transplant. None of these dyes stains the individual bacilli. These experiments therefore corroborate and amplify the results obtained by direct staining, and show that tubercle bacilli can grow even when in immediate contact with certain of the fat dyes. It may be recalled that Dr. Corper, in a previous article of this series, reported that the fat dyes had no influence upon the course of experimental tuberculosis in animals.

CONCLUSIONS.

All the dyes used, whether fat-soluble or not, stain pure cultures of tubercle bacillus, *en masse*, because of the presence of stainable substances outside the bacilli.

Sudan III does not stain individual tubercle bacilli, either in smears of pure culture, in tuberculous pus, or in tuberculous tissue.

Sudan yellow and sudan brown stain the bacilli, faintly, in pure culture smears, upon prolonged exposure, or on heating.

Scarlet R resembles sudan III in behavior, but is slightly less

inefficient, about half the tests for individual staining being doubtful or even faintly positive.

Nile blue sulfate gives a faint and rather unsatisfactory bacillus-stain, as does Janus green, for the most part. A single smear stained with Janus green showed deeply stained bacilli, but this could not be duplicated.

Indulin stains the bacilli faintly, upon prolonged application.

Indophenol blue does not show any bacillus-stain.

Dimethylaminoazobenzol gives a faint and unsatisfactory bacillus-stain.

Basic fuchsin, which is only slightly fat-soluble, eosin, and methylene blue, which are not fat-soluble, stain the individual bacilli deeply in a relatively short time.

All the dyes used stained the impure ether extract of tubercle bacilli, while the purified ether extract was less readily stained by the majority of the dyes not classed as "fat-dyes."

The behavior of the dyes toward the impure ether extract corresponds with their behavior toward cultures of the bacilli, and is very different from that toward the individual bacilli. These facts seem to indicate that masses of ether-soluble substance exist on the surfaces of cultures as well as within the bacterial protoplasm, and it is with this extra-cellular material that the dyes combine.

Basic fuchsin and eosin, and to a less extent Bismarck brown, resemble the regular fat-dyes in the ease with which they stain the ether extract.

Dilute solutions of Nile blue sulfate and neutral red are more efficient than the saturated, in the staining of the ether extract.

The "fat-dyes" are not serviceable for the detection of tubercle bacilli in pus or in tissue, nor for their staining in pure cultures.

Experiments with crushed bacilli confirm Benians' view that the acid-fastness of the tubercle bacillus depends upon the physical integrity of the bacterial cell.

The fatty constituents of the tubercle bacillus are not, *per se*, the cause of the staining reaction characteristic of this organism.

This work has been done under the direction of Dr. H. G. Wells, and with the direct supervision of Dr. Lydia M. DeWitt; and to both I make most grateful acknowledgment for suggestions and assistance.